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Abstract: It is proposed that metabolism of several structurally-related chemicals by CYP2F isoforms of the cytochromes P450 family results in a cytotoxicity-driven mode of action in organs high in CYP2F; namely, CYP2F2 in nasal and lung tissue in mice and CYP2F4 in nasal tissues in rats. Importantly, the CYP2F1 isozyme expressed in humans appears to have a low capacity to metabolize these compounds. In mice, the resultant cytotoxicity and subsequent regenerative hyperplasia is hypothesized drive an increase in lung tumors that are mostly benign and are not life shortening. Although a complete picture of the mode of action has not been developed in any one model compound, data from the individual compounds can be combined to synthesize and reinforce confidence in the CYP2F toxicity hypothesis. For coumarin, naphthalene, and styrene, inhibition of toxicity with inhibition of CYP2F2 has been demonstrated. Rat CYP2F4 appears to be equally active in metabolizing these chemicals; however, CYP2F4 occurs to a much lower extent in

rat Clara cells and levels of metabolites produced are not sufficient to cause lung cytotoxicity. Human lungs contain far fewer of Clara cells than rats or mice, and human lung microsomes failed to, or only marginally, metabolize these compounds. In addition, the human lung differs markedly from the mouse lung in the morphology of its Clara cells, which make humans much less sensitive than mice to toxicity due to reactive metabolites. The absence of a role for CYP2E1-generated metabolites (primarily alkyl oxidation vs. ring-oxidation) in mouse pulmonary effects was demonstrated by the lack of protection from styrene toxicity by CYP2E1 inhibitor, or reduction of toxicity in CYP2E1-knockout mice, and lack of lung toxicity of the primary metabolite of ethylbenzene. The chemicals used as examples of this mode of action generally are negative in standard genotoxicity assays. Apart from increased SCE, no consistent pattern in genotoxicity results was found among these chemicals. Thus, while lung tumors from bronchiolar cell cytotoxicity are theoretically possible in humans, it is unlikely that metabolism by CYP2F1 would produce levels of cytotoxic metabolites in human lungs sufficient to result in lung cytotoxic responses and thus tumors. Therefore, it is unlikely several chemicals that cause mouse lung tumors via CYP2F2 metabolism will cause lung tumors in humans.

*** Conflict of Interest Form**

Dr. Cruzan provides science consulting to the Styrene Information and Research Center (SIRC), Arlington, VA. Some of Dr. Carlson's research on styrene metabolism and toxicity has been funded by SIRC. The Dow Chemical Company (JB) has a financial interest in styrene, ethylbenzene, cumene, and divinylbenzene. LyondellBassell Industries (MB) has a financial interest in ethylbenzene and styrene. Shell Oil Company (RG) has a financial interest in styrene ethylbenzene, and cumene.

Signed conflict of interest statements will be supplied by all authors offline.

*** Cover Letter**

January 27, 2009

Editor, Regulatory Toxicology and Pharmacology

Dear Sir:

We are submitting the attached manuscript on a mouse lung tumor mode of action (MOA) for consideration in Regulatory Toxicology and Pharmacology. This MOA has been developed using data from several chemicals. We believe this provides an important tool in assessing species specific metabolic activation in tumorigenesis.

Thanks for your consideration.

George Cruzan, PhD, DABT

Mouse Specific Lung Tumors from cyp2f2-Mediated Cytotoxic Metabolism: An Endpoint/Toxic Response Where Data from Multiple Chemicals Converge to Support a Mode of Action

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Introduction

In 2005, the US EPA published revised Cancer Risk Assessment Guidelines. The Guidelines emphasize collection of all relevant data on the mode(s) of action of tumor formation in experimental animals, and an evaluation of their relevance to humans. The Guidelines provide a framework for evaluating modes of action (Section 2.4). In addition, other institutions have developed frameworks to organize datasets to better inform mode of action evaluations (Boobis et al., 2006; Seed et al., 2005). Mode of action is defined as identification of the key biological (precursor) events leading to adverse toxicities including cancer (EPA, 2005)

The EPA Guidelines emphasize invoking default assumptions only when there is insufficient data to explain a mode of action. A major uncertainty in the application of the Guidelines is deciding how much data are needed to establish a mode of action vs. reliance on pre-set default assumptions (Guyton et al, 2008; Guzelian et al., 2007). It is generally understood that mode of action evaluations are deliberately focused on identifying the key biological event(s) driving expression of toxicity. However, establishing the quantity and quality of data necessary to fulfill criteria for appropriate implementation of mode of action frameworks nonetheless remains a challenge. Several approaches recently have been suggested to facilitate weight-of-evidence data evaluations of potentially complex datasets (Rhomberg, 2007; Guzelian et al., 2005; Weed, 2005).

Reliance on the best understanding of the data, through weight-of-evidence approaches to identify key mode of action events, appears to represent the intent of the EPA Guidelines. The Internationale Program on Chemical Safety (IPCS) has expanded this framework to include a structured evaluation of the adequacy of data to explain the mode of action in animal models, the qualitative applicability to humans, and the quantitative relevance to human risk (Boobis et al., 2006).

Few examples of mode of action evaluations have been published (Gift, 2005; Boobis et al., 2006; Guzelian, 2007). For most of these evaluations, the spectrum of data considered was

largely restricted to data specifically available for the chemical under evaluation. However, it is clearly recognized that for some of the established modes of action, such as α-2_u-globulin-mediated male rat kidney toxicity (US EPA, 1991), data on the key event(s) from multiple chemicals greatly informed scientific and regulatory acceptance of the proposed modes of action.

A growing body of data is available indicating that a series of structurally related chemicals (Fig. 1) produce toxicity in the terminal bronchioles that results in subsequent late-developing bronchiolo-alveolar tumors in the lungs of mice, but not in rats (Table 1). These chemicals include coumarin (NTP, 1993a), naphthalene (NTP, 1992, 2000), styrene (Cruzan et al., 1998, 2001), ethylbenzene (NTP, 1999), cumene (NTP, 2007a), a-methylstyrene (NTP, 2007b), divinylbenzene (NTP, 2007c) and benzofuran (NTP, 1989). Some of these chemicals produced increased tumors at other sites in mice, and may act by multiple MOAs. Styrene (Cruzan et al., 1998, 2001) and divinylbenzene (NTP, 2007c) induced only lung tumors in mice and no tumors in rats. However, some of these chemicals increased tumors at other non-respiratory sites in mice, and thus may be acting by multiple MOAs apart from CYP2F-mediated metabolism in these target organs. Experimental observations across the datasets available for these compounds present an excellent opportunity to develop, evaluate and support a unifying mode of action hypothesis supporting the species-specific lung toxicity and carcinogenicity of this series of compounds. Not surprisingly, the extent of mode of action data for each of these chemicals is varied; some endpoints have been examined in more than one chemical, but few endpoints have been examined in all of the chemicals. However, the cumulative data available from the range of chemicals have contributed to an understanding of a common mode of action for the selective development of lung toxicity and subsequently tumors in mice. In the following sections, this mode of action is explored using the EPA and IPCS frameworks.

1. **Postulated MOA for Mouse-Specific Lung Toxicity and Tumorigenicity**

Metabolism of several chemicals by CYP2F2 in terminal bronchiolar Clara cells in mice results in the localized generation of cytotoxic metabolites and subsequent reparative cell replication. Initial exposures lead to cytotoxicity in terminal bronchioles, followed by reparative cell replication. On continued exposure, the increased cell replication leads to cellular crowding and

then to hyperplasia in the terminal bronchioles. As the hyperplasia continues, it expands into the surrounding alveolar ducts. Some of these hyperplasias proceed to form adenomas in the mouse lung. The chemical-induced hyperplasia elevates spontaneous incidence of adenomas in control mice [Table 1]. Depending on the severity of the stress, a few of the adenomas may progress to carcinomas. The analogous CYP2F4 in rats may be as capable of forming these cytotoxic metabolites as mouse cyp2f2; however, rats have much lower levels of CYP2F4 in terminal bronchioles and do not produce sufficient levels of these metabolites to cause cytotoxicity, hyperplasia, or lung tumors. Tissues that are high in CYP2F enzymes (CYP2F2 in mouse lung terminal bronchioles and nasal olfactory epithelium; CYP2F4 in rat nasal olfactory epithelium) develop cytotoxicity from these chemicals, which may or may not progress to tumors. Humans have very small amounts of the orthologous isozyme CYP2F1 in lungs or nasal turbinates. CYP2F1 appears to be much less, if at all, active in metabolizing these compounds. Therefore, no cytotoxicity or lung tumors are expected from human exposures to these chemicals. The key element of the hypothesis is that the lung-specific toxicity of this series of compounds converges on their metabolism to cytotoxic metabolites by mouse CYP2F2, which may differ in both specificity (nature of metabolites formed) and rate of metabolism compared to rats and humans. This is shown pictorially using styrene as an example in Fig. 2.

2. Key events

Key events in this MOA are: delivery of the chemical to the respiratory system, metabolism in lung, cytotoxicity in terminal bronchioles, cell replication, and tumors.

2.1. Delivery of Chemical to the Respiratory System

The respiratory system (nasal epithelium to alveoli) is the major interface between mammals and airborne chemicals in their environment. Inhalation of these chemicals delivers them directly to the cells lining the airways. Depending on the physico-chemical properties of the substance, at

very low concentrations as much as 50% of the inhaled chemical in the airstream can be absorbed in the nose (Morris, 2000). Uptake is highly dependent on localized metabolic capacity. In the presence of CYP metabolism inhibitors, about 10% of the styrene is absorbed in the nasal region (Morris, 2000) as compared to 50% of the styrene removed from the airstream in the upper respiratory tract of control mice. These chemicals can also be absorbed directly into the cells of the terminal bronchioles (Clara cells) as well as the alveolar cells. When absorbed into alveolar cells, they pass into the blood capillaries and are distributed systemically in rats and mice, resulting in detectable blood concentrations of the parent compounds (Cruzan et al., 1998, 2001).

For coumarin, naphthalene, styrene, and ethylbenzene, there is good evidence of distribution of the chemical from all routes of exposure to all tissues, including respiratory epithelium. When exposure is by the oral route, first pass metabolism in the liver dramatically reduces the amount of chemical that is distributed to tissues through the blood stream (Sarangapani et al., 2002). However, metabolism and cytotoxicity in lung from oral exposure has been demonstrated for coumarin (NTP, 1993), styrene (Green et al., 2001a), ethylbenzene (Stott et al, 2003), indicating that these chemicals can be absorbed systemically and distributed to all organs after oral administration, and that the lung has a capacity to metabolize systemically available concentrations of these compounds.

Qualitatively, delivery of these chemicals to lung cells does not appear to be species specific. This process is driven by the solubility of the chemicals in the various tissues, which should be approximately the same across species, including man and by blood flow and minute volume, which may affect the quantity of these chemicals delivered to the lungs.

2.2. Metabolism in Lung

Many compounds are metabolized to cytotoxic metabolites by CYP2F2 in the Clara cells of the terminal bronchioles of mouse lung. The metabolite(s) responsible for cytotoxicity from most of these compounds in the terminal bronchioles have not been determined.

Coumarin: The major metabolite of coumarin in rats, mice, and humans is 7-hydroxycoumarin. However, coumarin is metabolized by CYP2F2 to coumarin-3,4-epoxide in mouse lung, which rearranges to 2-hydroxyphenylacetaldehyde (Born et al., 2002) and causes mouse lung cytotoxicity and is believed to cause lung tumors. Inhibition of CYP2F2 by 5-phenyl-1-pentyne (5P1P) eliminated the bronchiolar cytotoxicity from coumarin (Born et al., 2002). This metabolism occurs to a much lower extent in rats, which do not develop lung cytotoxicity or lung tumors (Felter et al., 2006). 3,4-Dihydrocoumarin is not capable of forming the 3,4-epoxide and did not induce lung tumors in mice (NTP, 1993b).

Naphthalene: Pulmonary microsomes from mice metabolized naphthalene at approximately 8 times the rate of rat microsomes and produced mostly 1R,2S-naphthalene oxide, whereas rat microsomes produced mostly 1S, 2R- naphthalene oxide (Buckpitt et al., 2002). Inhibition of CYP2F2 by 5P1P eliminated the bronchiolar cytotoxicity from naphthalene (Chang et al., 1996). Genter et al. (2006) demonstrated that CYP1A1 and CYP1A2 genes which are inducible by AHR in the mouse respiratory tract do not function to influence naphthalene toxicity, and confirm the results of Phimister et al. (2004) that CYP2F2 bioactivates naphthalene in lung and nasal tissues.

Styrene: For styrene, the first step in the major metabolic pathway is oxidation to S-styrene-7,8-oxide; this accounts for at least 80% of the metabolism of styrene in rats and mice (Sumner and Fennell, 1994; Cruzan et al., 2002). It should be noted that oral administration of styrene-7,8-oxide to mice at 275 mg/kg/day did not result in increased lung tumors (Lijinski, 1986), even though PBPK models indicate this dose of SO would result in a higher lung level of SO than from metabolism of styrene at 40 ppm by inhalation (Sarangapani et al., 2002). Further, Hofmann et al. (2006) demonstrated that *ex vivo* exposure to styrene in rat lungs at 1000 ppm (non-tumorigenic) produced 2.5 nmol styrene oxide/g lung vs. 0.25 from mouse lungs at styrene concentration of 40 ppm (tumorigenic). This led the authors to conclude that styrene-7,8-oxide is not the agent responsible for mouse lung cytotoxicity from styrene exposure. In mouse lung, two alternate metabolic paths are prevalent; one involves formation of R-styrene-7,8-oxide and the other involves oxidation of the benzene ring (Cruzan et al., 2002; Bartels et al., 2005). Using

selective inhibitors, Carlson determined that CYP1A, 2B, and 2E1 had little, if any, impact on Clara cell cytotoxicity of styrene, implying they are not involved in metabolic activation of styrene in the lung (Carlson 1997, Carlson et al., 1998). Inhibition of 2E1, or the use of 2E1-knockout mice demonstrated that 2E1 plays some role in the acute liver cytotoxicity of styrene, but has no impact on the lung toxicity (Carlson, 2004; Vogie et al., 2004). In studies of styrene, the inhibition of CYP2F2 by 5-phenyl-1-pentyne (5P1P) inhibited both the lung and nasal cytotoxicity in CD-1 mice (Green et al., 2001a, b). 4-Vinylphenol (4-VP, 4-hydroxystyrene), although a minor urinary metabolite of styrene, has been examined for its lung toxicity potential. 4-VP is 10 times as toxic to mouse lung as styrene and 5 times as toxic as styrene-7,8-oxide (Carlson et al., 2002). Inhibition of CYP2F2 by 5P1P also inhibited the cytotoxicity of 4-VP (Carlson, 2002), indicating that there is a subsequent metabolite of 4-VP that is responsible for cytotoxicity. The metabolite(s) responsible for cytotoxicity from these compounds in the olfactory epithelium or terminal bronchioles have not been identified.

Ethylbenzene: *In vitro* studies examining comparative mouse, rat and human lung and liver microsomal metabolism of ethylbenzene have confirmed extensive metabolism in all three species to alkyl-oxidized metabolites, e.g., 1-phenylethanol (mouse > rat ~ human; Saghir et.al., 2006; 2008). 1-Phenylethanol was not pneumotoxic or tumorigenic when tested in high-dose oral subchronic and chronic rat and mouse bioassays (NTP, 1990). No detectable lung toxicity was found from exposure to 1-phenylethanol, 2-phenylethanol, or phenylacetaldehyde in mice (Carlson et al., 2002). However, in vitro studies suggested possible formation of potentially lung-toxic reactive ring-oxidized metabolite(s) (Saghir et.al., 2008). Use of GSH-trapping to detect putative cytotoxic catechol and hydroquinone metabolites confirmed the *in vitro* formation of these metabolites in mouse, rat and human liver microsomes, and in mouse and rat, but not human, lung microsomes. Similar to the generation of alkyl-oxidized metabolites, mouse lung microsomes exhibited substantially higher metabolic activity (mouse lung GSH-derived metabolites approximately 10X > rat lung; human lung not detectable; mouse lung GSH metabolites approximately 2X > mouse liver; mouse liver approximately 10X > rat and human liver). Although ring-oxidized metabolites accounted for a relatively small fraction of overall ethylbenzene metabolism, their selective elevation in mouse lung microsomes is nonetheless consistent with the hypothesized mode of action attributing preferentialformation of lung-derived

cytotoxic, ring-oxidized metabolites as driving the mouse lung specific toxicity of ethylbenzene. Interestingly, both mouse and rat lung microsomes exhibited decreasing amounts of ring-oxidized metabolite formation with increasing concentrations of ethylbenzene, suggesting the possibility of cytochrome P450 suicide inhibition by reactive ring-oxidized metabolite(s). This observation would also be consistent with the hypothesis of the formation of reactive cytotoxic metabolites in mouse lung. Studies of the inhibition of the CYP2F2 metabolism of ethylbenzene by 5P1P are currently in progress. 4-Hydroxyethyl-benzene is the only metabolite of ethylbenzene that has been demonstrated to cause mouse lung cytotoxicity in 3-day studies (Kaufmann et al., 2005).

Cumene: In mice exposed to 14C-cumene, urinary metabolites included 4-(2-hydroxy-2-propyl)phenylsulfate, indicating ring oxidation (Ferguson et al., 2008).

Data on the metabolism of these compounds in human lung tissue are limited because of the difficulty obtaining adequate specimens for testing. However, limited data indicate that these metabolites are either not produced in human lung or are produced to a much lower degree (Vassallo et al., 2004; Buckpitt et al., 1986; Cruzan et al., 2002; Felter et al., 2006, Carlson, personal communication). Studies on CYP2F1 metabolism of specific compounds has been reported by a few different investigators, but there is no comparison of metabolic rats using the same conditions and CYP2F1 preparation.

Thus human lung and nasal cells would not be expected to develop cytotoxicity from these compounds.

2.3. Cytotoxicity

Short term exposure to coumarin (Born et al., 1998), naphthalene (West et al., 2001), styrene (Cruzan et al. 2002), and ethylbenzene (Stott et al., 2003) all cause cytotoxicity in the terminal bronchioles of mouse lung, but not rat lung (Table 2). The target cells are the Clara cells lining the terminal bronchioles. Toxicity to alveolar cells does not occur. Single gavage doses of 150 and 200

mg/kg coumarin resulted in swelling and necrosis of Clara cells in the terminal bronchioles of male and female B6C3F1 mice (Born et al., 1998). Doses below 150 mg/kg did not cause toxicity. While coumarin caused mouse lung cytotoxicity and lung tumors (NTP, 1993a), dihydrocoumarin did not (NTP, 1993b). Coumarin (NTP, 1993) causes cytotoxicity in the terminal bronchioles, but since it was administered orally the olfactory epithelium was not examined.

Naphthalene: The cytotoxicity from naphthalene is summarized by Buckpitt and coworkers (2002). Briefly, parenteral administration of 50 mg/kg naphthalene results in swelling of the Clara cells (O'Brien et al., 1985); larger doses result in more severe effects, including a loss of apical blebs and decreased endoplasmic reticulum in Clara cells and denuding of Clara cells from the terminal bronchioles. For naphthalene, female mice are more susceptible than males. CYP2F2 bioactivates naphthalene in mouse lung terminal bronchiolar tissue to one or more reactive metabolites that induce cytotoxicity after depleting glutathione (Phimister et al., 2004; Genter et al., 2006). In rats, even at an ip dose of 1600 mg/kg, the Clara cells were apparently normal.

Styrene: The cytotoxicity of styrene has been summarized by Cruzan et al. (2002, 2005). For styrene, cytotoxicity has been measured by increased cell replication following 3 inhalation (40 and 160 ppm) or ip (100 mg/kg) exposures (Green et al., 2001a; Kaufmann et al., 2005). Similarly, following 3 exposures, styrene metabolites styrene-7,8-oxide (100 mg/kg) and 4-hydroxystyrene (35 mg/kg), produced a greater increase in cell replication than the parent compound styrene (Kaufman et al., 2005). In the chronic mouse study (Cruzan et al., 2001), decreased staining of the Clara cells (an indicator of cytotoxicity) was reported in 50-70% of the mice exposed to 20 ppm for 12, 18 or 24 months and in more than 80% of those exposed to 40, 80, or 160 ppm. Increased cell proliferation has been reported at concentrations of 40 ppm or greater (20 ppm has not been examined). Bronchiolar hyperplasia was seen in a few mice exposed to 40 ppm for 12 months and in most mice exposed to 80 or 160 ppm; by 24 months bronchiolar hyperplasia was seen in up to 40% of the mice exposed to 20 ppm and in more than 75% of those exposed to 40, 80 or 160 ppm (Cruzan et al., 2001). Green and coworkers

demonstrated that metabolism of styrene by CYP2F2 is necessary to cause the cytotoxicity (Green et al., 2001a).

Ethylbenzene: Exposure of B6C3F1 mice to tumorigenic 750 ppm ethylbenzene exposures resulted in significantly increased S-phase DNA synthesis in the small airways after 1 week treatment (measured by BrdU incorporation); S-phase synthesis remained elevated after 4 weeks of exposures (non-significant approximate 2X increase; Stott, 2003). In addition, a re-evaluation of the mouse lung tissues from the ethylbenzene bioassay identified the presence of multifocal bronchiolar/parabronchiolar hyperplasia at the 750 ppm tumorigenic exposure level (Brown, 2000).

Glutathione Depletion: Consistent with formation of putative cytotoxic metabolite(s), administration of these chemicals results in GSH depletion. Phimister and coworkers (2004) demonstrated that administration of naphthalene resulted in GSH depletion. They further reported that lung GSH depletion precedes cellular injury, that lung GSH is depleted by levels of naphthalene that do not deplete liver GSH, and that liver GSH is not able to maintain lung GSH at normal levels following naphthalene administration. Carlson and coworkers have demonstrated glutathione (GSH) depletion in lung of mice administered 200 mg/kg styrene ip, which lasted through 6 hours, but returned to normal levels by 12 hours (Turner et al., 2005).

2.4. Cell replication

The mouse terminal bronchioles respond to the cytotoxic injury by generating replacement Clara cells. Increased cell labeling after short-term exposure has been demonstrated for styrene, naphthalene, ethylbenzene, and coumarin. Long-term exposure results in continued bouts of cytotoxicity and cell replication. Continually elevated cell replication leads to overproduction of Clara cells, leading to cellular crowding, followed by hyperplasia which can eventually extend into alveolar ducts (Cruzan et al., 2001). No increase in cell replication rates have been found in alveolar cells of mouse lungs from any of these compounds. No increase in cell replication rates was found in the lungs of rats exposed to styrene or ethylbenzene.

2.5. Tumors

For coumarin (NTP, 1993), naphthalene (NTP, 1992), styrene (Cruzan et al., 2001), ethylbenzene (NTP, 1999), cumene (isopropylbenzene) (NTP, 2007a), alpha-methylstyrene (isopropenylbenzene) (NTP, 2007b), divinylbenzene (NTP, 2007c) and benzofuran (NTP, 1989), lung tumors were increased in mice, but not in rats. Tumors were found in the outer layer of the lung where the terminal bronchioles and alveoli intersect. Tumors generally encompass areas of alveoli and bronchioles and are termed "bronchioloalveolar adenomas" or "alveolarbronchiolar adenomas", depending on the pathologist. For all the chemicals in this class, tumors occurred late in life and were not life-shortening; i.e., increased tumors were found only at study termination. In general, the increases were in benign tumors. In the case of styrene, increased lung tumors were found only at the end of the 24-month study, but not at the 12 and 18 month interim sacrifices (Cruzan et al., 2001); other studies did conduct interim sacrifices).

The incidence of lung tumors was not increased in mice exposed to 3,4-dihydrocoumarin (not able to form 3,4-epoxide), 4-methylstyrene (not able to form 4-hydroxystyrene), mixture of 3- and 4-methylstyrene (vinyltoluene, not able to form 3- or 4-hydroxystyrene), styrene-7,8-oxide, or 1 phenylethanol (side-chain oxidation product of ethylbenzene). These data suggest that ring oxidized metabolites of the parent compounds are critical to mouse lung tumor formation.

3. Adequacy of Evidence of MOA in animals

3.1. Strength of Association

Chronic inhalation exposure of ethylbenzene, styrene, naphthalene, cumene, α-methylstyrene, divinylbenzene, and coumarin have all been shown to increase the incidence of lung tumors among mice, but not rats. Cytotoxicity and increased cell replication were detected in coumarin, naphthalene, styrene, and ethylbenzene; in mice, all four cause terminal bronchiolar cytotoxicity

and increased cell replication at exposure levels comparable to the tumorigenic levels (Table 2). For coumarin, naphthalene, and styrene, it has been demonstrated that inhibition of CYP2F2 inhibits the cytotoxicity and cell replication. Structurally similar chemicals (3,4-dihydrocoumarin, 3-, or 4-methylstyrene) that cannot be oxidized by CYP2F2 to active ring-oxidized intermediates did not cause cytotoxicity or mouse lung tumors. Other chemicals have not been tested.

3.2. Consistency of Association

Cytotoxicity from these chemicals occurs in organs with high levels of CYP2F family. CYP2F2 (mouse) is expressed largely in Clara cells in the lung airways (most notably in the terminal bronchioles) and in the nasal olfactory epithelium, with little or none present in the liver. Extensive research has shown that there is a strong association between CYP2F expression levels and tissue susceptibility to naphthalene cytotoxicity (Buckpitt et. al., 2002). Styrene (Cruzan et al., 1997, 2001), naphthalene (NTP, 1992), cumene (NTP, 2007a), and alpha-methylstyrene (NTP, 2007b) cause cytotoxicity in the terminal bronchioles and nasal olfactory epithelium in mice. Ethylbenzene causes cytotoxicity in the terminal bronchioles, but not in the nasal olfactory epithelium at the concentrations tested (NTP, 1999). In rats, CYP2F4 is expressed mainly in the nasal olfactory epithelium, with lesser amounts in the lung. Styrene (Cruzan et al., 1997, 1998), naphthalene (NTP, 2000), cumene (NTP, 2007a), and alpha-methylstyrene (NTP, 2007b) cause cytotoxicity in the nasal olfactory epithelium of rats, but not in the lung terminal bronchioles. Ethylbenzene and coumarin do not cause cytotoxicity in either lung or olfactory epithelium in rats (NTP, 1999). Coumarin does not cause cytotoxicity in rat lung or nasal olfactory epithelium. In humans, CYP2F1 is expressed at very low levels in the lung, much lower than CYP2F4 in the rat. Therefore, it is not surprising that these chemicals have not been reported to cause cytotoxicity in human lung cells.

3.3. Specificity of Association

Mice have a much greater number of Clara cells than do rats, which in turn have a much greater number than humans. In addition, mouse Clara cells have much more CYP2F2 than the amount of CYP2F4 found in rat Clara cells. Human lung Clara cells have barely detectable levels of CYP2F1. Thus mice have the greatest number of target cells for toxicity, and those target cells

have the greatest capacity to produce toxic metabolites.

Toxicity in mice occurs in 2 organs which contain high levels of CYP2F2: nasal olfactory mucosa (chronic cytotoxicity, limited cellular replacement, cells replaced with respiratory-like cells), and lung (chronic cytotoxicity, rapid cellular replacement in kind, hyperplasia). Toxicity in both olfactory mucosa and Clara cells is prevented if CYP2F2 is inhibited by 5P1P. In rat lung and liver, with very little CYP2F4, these chemicals are metabolized primarily via CYP2E1. Rat nasal olfactory tissue contains a large amount of CYP2F4, in addition to CYP2E1 (Green, 2001b). In rat nasal olfactory tissue, large amounts of the toxic metabolites from these compounds are formed and cytotoxicity is seen from many of them.

4. Qualitative Relevance of the Animal MOA for Humans

The key events for this mouse lung tumor MOA are presented in Table 2 and Figure 2 (styrene as model toxicant).

Lung tumors are quite prevalent in humans, mostly related to cigarette smoking. These are thought to arise from bronchiolar cells and may involve cytotoxicity, as well as genotoxicity. This suggests that cytotoxicity in bronchioles of humans from chemicals could contribute to the formation of lung tumors.

The MOA proposes that the toxic effects in mice are due to preferential and lung-specific metabolism by CYP2F2. Rats have lower levels of CYP2F4 in terminal bronchioles and do not produce sufficient metabolites to cause cytotoxicity or lung tumors. Humans have much lower amounts of CYP2F1 and one would expect they would produce much lower levels of cytotoxic metabolites than mice or even rats (which do not exhibit tumor response). If human CYP2F1 or potentially some other CYPP450 could produce sufficient metabolites from a chemical sufficient to produce bronchiolar cell cytotoxicity, it could conceivably lead to lung tumors

5. Quantitative Relevance of the Animal MOA for Humans

Qualitative impacts of the proposed MOA on tumor outcomes are not fully defined and thus cannot be definitively excluded as a contributor to the hypothesized MOA. However, important quantitative differences between mice, rats and humans clearly exist and include: (1) Rodent exposures in the bioassays are orders of magnitude higher than expected human exposure; (2) Mouse lung has a larger fraction than the human lung with respect to Clara cells (Plopper et al., 1980a, b); (3) Rates of metabolism for these chemicals in lung microsomes exhibit clear species differences, with rates in mice being significantly greater than the corresponding rates in humans (Green et a., 2001; Vassallo *et al.*, 2004; Saghir *et al.*, 2006) and (4) Background rates for lung tumors are higher in male mice (~14%) than in humans (~7%, SEER, 2006). Given these species differences, the MOA is assumed to be minimally plausible in humans, but humans are expected to be substantially less sensitive than mice to the pulmonary effects of these chemicals. Because rat lungs contain more CYP2F4 than human lungs contain CYP2F1 and rats do not develop cytotoxicity or lung tumors from these chemicals, it is very unlikely that any chemical that causes mouse lung tumors by this MOA and does not cause rat lung tumors will cause human lung tumors.

6. Other potential modes of action

6.1 Postulated Alternative MOA: Genotoxicity

Another mode of action that must be considered is direct genotoxicity. For styrene and divinylbenzene, mouse lung tumors are the only tumorigenic response in rats or mice. For naphthalene, only lung tumors in mice and nasal tumors in rats (also high in CYP2F) were found. For the remaining chemicals of this group, other tumors were increased in mice and/or rats. Finding increased tumors in multiple organs and/or species leads to a suspicion of a genotoxic MOA.

6.2. Key Events for Genotoxic MOA

Standard mutagenicity studies are largely negative (Table 3). Nearly all Ames assays were negative, while mouse lymphoma tests produced 1 negative (ethylbenzene), 1 positive (benzofuran) and 1 equivocal (ethylbenzene) result. Mutations at the hprt locus were negative for cumene in CHO cells and positive or negative for styrene in CHL cells. *In vitro* Unscheduled DNA synthesis (UDS) assays were mixed for cumene (1 positive, 1 negative) and negative for ethylbenzene.

Positive results in *in vitro* chromosomal aberration assays (Table 3) were reported for styrene and coumarin, while negative results were reported for α-methylstyrene, ethylbenzene and benzofuran. Positive results in *in vitro* micronucleus assays (Table 3) were reported for styrene and naphthalene; the others have not been tested. Positive results in *in vitro* SCE assays (Table 3) were reported for styrene, naphthalene, benzofuran and α-methylstyrene; the others have not been tested.

In vivo chromosomal aberration assays in laboratory animals (Table 4) are largely negative for styrene (1 positive, 11 negative); none of the others have been tested *in vivo*. *In vivo* micronucleus assays (Table 4) are largely negative for coumarin, styrene, naphthalene, ethylbenzene, cumene, α-methylstyrene, and divinylbenzene. An *in vivo* unscheduled DNA synthesis (UDS) assay was negative for ethylbenzene. SCE has been examined in laboratory animals only for styrene; 6 assays were positive and 1 was negative (Table 4). Styrene was not genotoxic in mouse lung in the two *in vivo* tests in which it was studied: lung tumor initiation in A/J mice (Brunnemann et al., 2002); chromosomal aberrations in B6C3F1 mice by inhalation (Kligerman et al., 2002).

A large number of studies of genotoxicity endpoints have been reported for reinforced plastics workers (Table 5), who are exposed to styrene in the workplace. No studies of genotoxic endpoints have been conducted in workers exposed primary to the other compounds. The reinforced plastics worker studies are subject to different interpretations. All reviewers agree that there are many studies with small numbers of subjects and/or controls, controls may not be well matched, and techniques may not have followed guidelines. Scott and Preston, 1994, Bonassi et al. 1996, and Henderson and Speit, 2005 concurred that there was not reliable evidence that

styrene exposure caused increased micronuclei in these workers. Scott and Preston, 1994 and Henderson and Speit, 2005 concluded that there was no clear evidence of increased chromosomal aberrations in these workers, while Bonassi et al., 1996 concluded that workers exposed above 30 ppm had increased aberrations. IARC (1994) listed 8 studies of chromosomal aberrations in these workes as positive and 12 as negative, while a recent draft on styrene by the NTP concluded that 4 of these negative studies should be considered positive. Improved industrial hygiene in the reinforced plastics industry further complicates interpreting these studies. Prior to 1980, exposures were normally up to 100 ppm average; improved conditions and the use of respiratory protection have reduced worker exposure to less than 50 ppm. About two-thirds of the chromosomal aberration studies in styrene-exposed workers published before 1990 were negative, while about 50% of those published since 2000 are positive, the opposite of the exposure trend. About half of the studies of SCE in these workers are reported as positive.

Interaction with DNA has been demonstrated for some of these chemicals. Low levels of DNA adducts have been reported in rats and mice exposed to styrene, but the levels are not higher in the target species (mouse) or target organ (lung Clara cells) (Boogaard et al., 2000; Cruzan et al., 2002). Adducts of naphthalene to hemoglobin, albumin, and other proteins have been reported (IARC, 2002). Tests for DNA or protein adducts have not been conducted for the other chemicals.

DNA strand breaks, or abasic sites have been found following exposure to styrene (IARC, 1994; 2002) and naphthalene (IARC, 2002). Tests for DNA strand breaks/abasic sites have not been performed on the other chemicals.

6.3. Adequacy of Evidence of MOA in animals

6.3.1. Strength of Association

Metabolites of several of these chemicals interact with cellular macromolecules including DNA. Only weak associations with mutational endpoints, if any, have been reported in *in vitro* studies

and even less so in *in vivo* studies in laboratory animals.

6.3.2. Consistency of Association

The genotoxicity data for these chemicals are inconsistent. There are indications of reactive metabolites that can interact with DNA, and there are some positive results, particularly in *in vitro* studies. However, the *in vivo* studies in using controlled and frequently high exposures to laboratory animals do not indicate mutagenicity or clastogenicity, but there may be increased SCEs. There is no consistent finding in any test of positive genotoxicity results across the chemicals tested.

6.4. Qualitative Relevance for of this Proposed Animal MOA for Humans

If metabolites of these chemicals cause lung tumors in mice via a genotoxic MOA in animals, those metabolites potentially could also cause tumors in humans via the same genotoxic MOA.

6.5.Quantitiative Relevance of this Proposed Animal MOA for Humans

Whether the lung tumors are caused by genotoxic reactions or cytotoxic reactions of metabolites, tumors will likely not result in the absence of generation of sufficient amounts of cytotoxic metabolites serving as a promoting driver. Since human CYP2F1 is barely detectable, and the chemicals studied to date appear to be poor substrates for this isozyme, lung tumors are extremely unlikely in humans.

7. Uncertainties, Inconsistencies, and Data Gaps

7.1. Uncertainties

The mode of action is characterized by the formation of cytotoxic metabolites by CYP2F2 in Clara cells. For coumarin, it is proposed that this metabolite is 2-hydroxyphenylacetaldehdye. Ring oxidized metabolites have been demonstrated to be the only cytotoxic metabolites for ethylbenzene and are much more toxic than side-chain only metabolites for styrene. Further

studies on the toxic metabolites from these chemicals may reduce the uncertainty of the qualitative and quantitative relevance of this mode of action for human risk.

Human lung tissue is difficult to obtain and study. Human lung preparations often are from diseased persons and the health status of the samples may be compromised. Further, cytochrome P450 activity of tissue samples or microsomes prepared from these tissues lose their catalytic activity quite rapidly. In addition, CYP2F seems to be located only in Clara cells and humans have fewer Clara cells than mice; thus, when using "general" lung tissue, the activity in human preparations is expected to be lower than in mouse preparations.

The cytotoxic effects of these compounds have been demonstrated primarily in *in vivo* experiments in mice. Limited *in vitro* data have shown toxic effects in Clara cells isolated from mice for styrene and metabolites, but the results are not consistent with the *in vivo* effects (styrene is equally toxic as styrene oxide and 4-vinylphenol). At present it is not known if these inconsistencies are attributable to dose deliver issues associated with these *in vitro* approaches. Effective examination of *in* vitro metabolism and/or toxicity in human lung tissue is likely to prove very challenging in that healthy and metabolically-active human lung tissue is difficult to obtain.

Confirmation of the necessity of CYP2F2-mediated metabolism to the cytotoxicity of these compounds may be obtained by administration to CYP2F2-knockout mice. Development of these CYP2F2-knockout mice is currently underway.

While the metabolism and cytotoxicity of these compounds cannot be studied in humans, there is a program underway to develop mice where the mouse CYP2F2 gene has been replaced by the human CYP2F1. These mice should be resistant to cytotoxicity if human CYP2F1 is indeed not capable of generating sufficient reactive metabolites from these chemical substrates. Thus a lack of cytotoxicity in these mice would provide strong evidence that humans are not sensitive to the

development of lung tumors by this mode of action. These mice could also be used to quantify the degree of metabolism of these compounds by the human CYP2F1 enzyme.

7.2. Inconsistencies

For most of the chemicals tested, effects were seen only at the highest doses tested. For some increased lung tumors were found only in males, while for others tumors were found only in females, and for others they were noted in both sexes. It is suspected that these dose and sex-specific differences may be attributable the pragmatic ability of the animals to equally tolerate comparatively high doses of chemical.

For most of the chemicals, cytotoxicity was also seen in the nasal olfactory epithelium of mice and rats. One exception was ethylbenezene; this differentiation may be due physico-chemical properties of ethylbenzene, its potential substrate affinity for CYP2F and/or other factors. It is suspected that a higher concentration would produce the nasal lesions. Coumarin was administered by gavage, but cytotoxicity was not seen in the nasal tissue. This may be because gavage administration is unable to deliver sufficient coumarin to the nasal to cause cytotoxicity.

7.3. Data Gaps

It is proposed that there are sufficient data across this series of lung-toxic compounds to support this mode of action. Further studies in CYP2F2-knockout mice may provide confirmatory evidence, but are not required to accept this mode of action.

8. Conclusions

It is proposed that metabolism of several chemicals by CYP2F family results in cytotoxicity in organs high in CYP2F; namely, CYP2F2 in nasal and lung tissue in mice and CYP2F4 in nasal tissue in rats. In mouse lung, an organ with a high background incidence of tumors, the resultant

cytotoxicity and subsequent regenerative hyperplasia leads to an increase in lung tumors that are mostly benign and are not life-shortening. A complete picture of the mode of action has not been developed in any one model compound, but is synthesized using data from several chemicals (Table 6; shown pictorially using styrene as an example in Fig. 2). Some chemicals are included because they have similar structures, and the bioassay data fit the pattern. Other chemicals are included because metabolism by CYP2F2 has also been demonstrated. For coumarin, naphthalene, and styrene, inhibition of CYP2F2 resulting in inhibition of toxicity in has also been demonstrated.

The chemicals used as examples of this mode of action generally are negative in standard genoxicity assays. However, the more assays that have been conducted on a chemical, the more likely that some individual assays would yield positive responses. The examples are generally negative in the Ames assay; two were tested in mouse lymphoma – 1 positive, one equivocal. *In vitro* chromosome aberrations or micronuclei were assayed in 6 chemicals; 4 were negative, 1 equivocal and 2 positive. *In vivo* chromosome aberrations or micronucleus were tested in 7 of the 8 chemicals, all were negative. Sister chromatid exchange was positive in all 4 chemicals tested *in vitro* and in the only one tested *in vivo*.

Oxidation of several chemicals to cytotoxic metabolites by the Clara cell specific enzyme CYP2F2 appears to be a biologically plausible MOA for mouse lung tumors. The orthologous rat CYP2F4 appears to be equally active in metabolizing these chemicals; however, CYP2F4 occurs to a much lower extent in rat Clara cells and levels of metabolites produced are not sufficient to cause cytotoxicity. Also, human lungs contain far fewer numbers of Clara cells than mice (Plopper et al., 1992; Stott *et al.*, 2003), and human lung microsomes failed to or only marginally metabolized these compounds (Saghir *et al.*, 2006; Green et al., 2001, Carlson; Vassallo *et al.*, 2004). In addition, the human lung differs markedly from the mouse lung in the morphology of its Clara cells, which make humans much less sensitive than mice to toxicity due to reactive metabolites (Green, 2000). The consistency of the relative species differences across chemicals with similar modes of action suggests that the decreased activity in human respiratory tract is not due to viability issues associated with explanted human tissues, but instead reflects a

fundamental species difference with respect to the distribution, expression, and/or activity of CYP2F in the respiratory tract. The absence of a role for CYP2E1 metabolites (a significant contributor to alkyl oxidation) in mouse pulmonary effects was demonstrated by the lack of protection from styrene toxicity by CYP2E1 inhibition or reduction of toxicity in CYP2E1-knockout mice (Carlson, 2004).

Therefore, while this mode of action is theoretically possible in humans if sufficient metabolites are produced, this is highly unlikely to occur, and these chemicals are not expected to cause lung tumors in humans.

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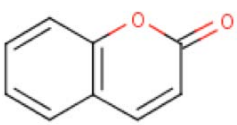
Legends to Figures:

Figure 1. Chemical structure of Chemicals Included in the Assessment of cyp2f2-Mediated Mouse Lung Tumors.

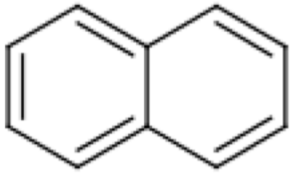
Figure 2. Postulated MOA for cyp2f2-Mediated Mouse Lung Tumors. The postulated MOA is illustrated using styrene. The major metabolic pathway in liver and lung via cyp2e1 metabolism to styrene-7,8-oxide, but lung cytotoxicity and tumors result from the production of different metabolites via cyp2f2 oxidation.

Figure 1

Figure 1. Formulas for Chemicals Included



Coumarin



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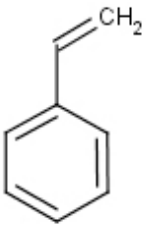

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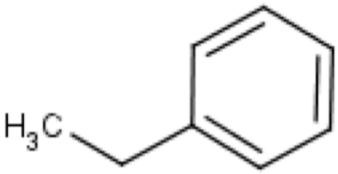

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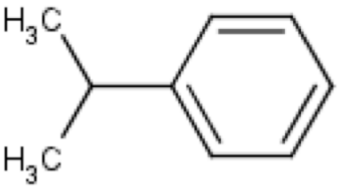
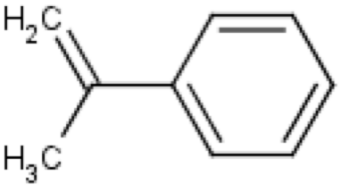
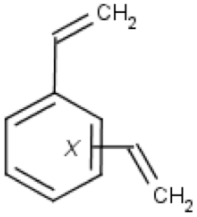


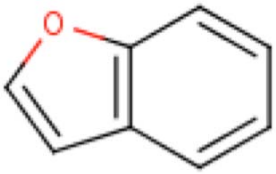
Cumene



alpha-Methylstyrene



Divinylbenzene



Benzofuran

Figure 2

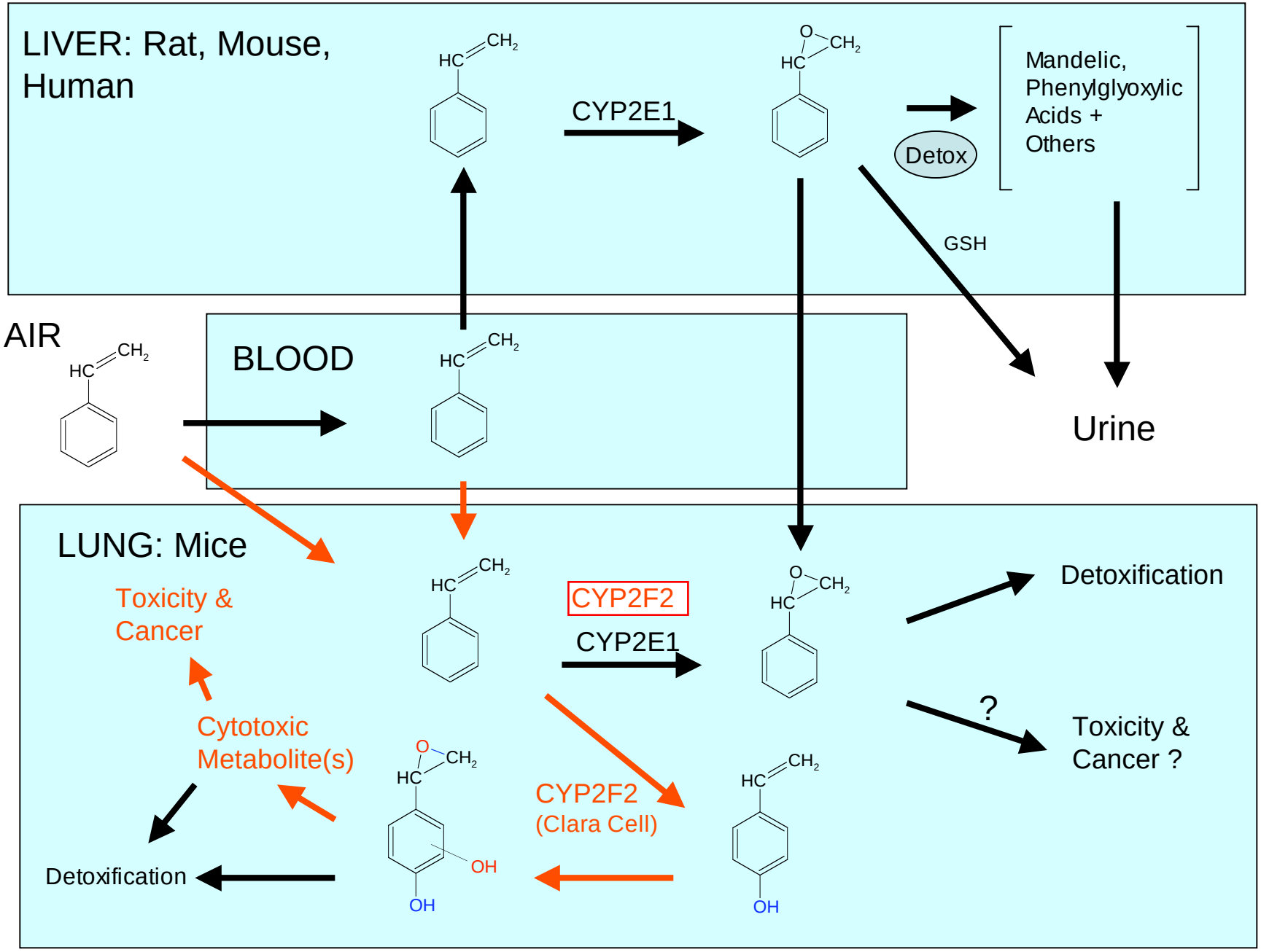

LIVER: Rat, Mouse, Human
CYP2E1
Detox
Mandelic, Phenylglyoxylic Acids + Others
GSH
Urine
AIR
BLOOD
LUNG: Mice
Toxicity & Cancer
CYP2F2
CYP2E1
Detoxification
Cytotoxic Metabolite(s)
?
Toxicity & Cancer ?
CYP2F2 (Clara Cell)
Detoxification
OH
OH
OH

Table 1

Table 1. Examples of Chemicals that Cause Mouse, but not Rat, Lung Tumors

Chemical	Exposure Route and Dose (Strain)	Lung Tumors in Male Mice	Lung Tumors in Female Mice
Coumarin	Oral: 0, 50, 100, 200 mg/k/day (B6C3F1)	14/50; 8/50; 14/50; 25/50	2/51; 5/49; 7/49; 27/51
Naphthalene	Inhalation: 0, 10, 30 ppm (B6C3F1)	7/70; 17/69; 31/135	5/69; 2/65; 28/135
Styrene (ethenylbenzene)	Oral: 0, 150, 300 mg/kg/day (B6C3F1) Inhalation: 0, 20, 40, 80, 160 ppm (CD-1)	0/20; 6/50; 9/50 17/50; 24/50; 36/50; 30/50; 36/50	0/20; 1/50; 3/50 6/50; 16/50; 17/50; 11/50; 27/50
Ethylbenzene	Inhalation: 0, 75, 225, 750 ppm (B6C3F1)	7/50; 10/50; 15/50; 19/50	4/50; 6/50; 5/50; 8/50
α-Methylstyrene (isopropenylbenzene)	Inhalation: 0, 100, 300, 600 ppm (B6C3F1)	13/50; 6/50; 9/50; 9/50	2/50; 7/50; 5/50; 6/50
Cumene (isopropylbenzene)	Inhalation: 0, 250, 500, 1000 ppm (B6C3F1)	19/50; 38/50; 42/50; 43/50	4/50; 31/50; 42/50; 46/50
Divinylbenzene	Inhalation: 0, 10,30, 100 ppm (B6C3F1)	16/50; 10/50; 8/50; 20/50	6/50; 12/50; 8/50; 13/50
Benzofuran	Oral\: 0, 30, 60 mg/kg/day	10/50; 9/40; 19/50	2/50; 9/50; 14/50

	(B6C3F1)		

Table 2. Dose and Temporal Relationships of Key Events in Mice

Chemical	Metabolism by CYP2F2	Acute Cytotoxicity	Sustained Cytotoxicity	Hyperplasia	Tumors
Styrene	yes	40 ppm*	20 ppm	160 ppm 3 months to 20 ppm after 2 years	Only at 2 years: 40 ppm - males and 20 ppm - females
Ethylbenzene	yes	750 ppm	750 ppm	750 ppm	750 ppm males only
Naphthalene	yes	8 ppm	30 ppm	30 ppm	30 ppm females only
Cumene	Not tested	Not tested	250 ppm males 125 ppm females	250 ppm males 125 ppm females	250 ppm males 125 ppm females
α-Methylstyrene	Not tested	Not tested	300 ppm females	300 ppm females only	100 ppm females only (not significant)
Divinylbenzene	Not tested	Not tested	10 ppm males and females	10 ppm males and females	10 or 100, not 30 females only

Coumarin	yes	150 mg/kg by gavage	None reported	None reported	200 mg/kg/day gavage males and females 275 mg/kg/day in diet – no increase
Benzofuran	Not tested	Not tested	120 mg/kg by gavage	120 mg/kg	120 mg/kg males and females

*lowest concentration tested

Table 3

Table 3. Summary of *In Vitro* Genotoxicity Studies

Ames	Cumene	negative 2 assays	Simmon et al., 1977; Florin et al., 1980
	Divinylbenzene	negative 3 assays	NTP, 2007c
	α-Methylstyrene	negative 1 assay	NTP, 2007b
	Coumarin	negative 2 assays	Lake et al., 1999
	Ethylbenzene	negative 4 assays	Dean *et al.,* 1985; Florin *et al*., 1980; Nestmann *et al*., 1980; Zeiger *et al*., 1992; Nestmann and Lee, 1983
	Benzofuran	negative 1 assay	NTP, 1989
	Naphthalene	negative 12 assays	See IARC, 2002
	Styrene	TA98 negative 13; TA100 positive 1, negative 13; TA1535 positive 4, negative 10; TA1537 negative 14; TA1538 negative 14	See IARC, 1994
Mouse lymphoma	Ethylbenzene	equivocal 2 assay; negative 1 assay	McGregor *et al*., 1988; Wollny, 2000; Seidel *et al*., 2006
	Benzofuran	positive 1 assay	NTP, 1989
CHO HPRT	Cumene	negative 2 assays	Yang, 1987; ; Gulf Life Sciences Center, 1985a
CHL, V79, hprt	Styrene	positive 1 assay negative 1 assay	IARC, 1994
UDS	Cumene	positive 1 assay; negative 1 assay	Curren, 1992; Gulf Oil, 1984b
Chromosomal aberrations	α-Methylstyrene	negative 1 assay	NTP, 2007b
	Coumarin	equivocal (positive only above 10 mM)	
	Ethylbenzene	negative 2 assay	Dean *et al.*, 1985; NTP, 1999
	Benzofuran	negative 1 assay	NTP, 1989
	Styrene	positive in 8 assays; negative in 1	See IARC, 1994
Micronucleus	Naphthalene	positive 1 assay	Sasaki et al., 1997
	Styrene	positive 2 assays	See IARC, 1994
Cell transformation	Cumene	negative 1 assay; positive 1 assay	Putnam, 1987); Gulf Oil, 1984a
	Naphthalene	negative 2 assays	Freeman et al., 1973; Rundell et al., 1983
Sister chromatid exchange	α-Methylstyrene	positive 1 assay	NTP, 2007b
	Benzofuran	positive 1 assay	NTP, 1989
	Naphthalene	positive 1 assay; negative 1 assay	Galloway et al., 1987; Tingle et al., 1993

	Styrene	positive 10 assays; negative 2 assays	See IARC, 1994; IARC, 2002

Table 4. Summary of *In Vivo* Genotoxicity Studies In Laboratory Animals

Micronucleus	Cumene	negative 2 assays, equivocal 1 assay	Gulf Life Sciences Center, 1985b; NTP, 2007a
	Divinylbenzene	negative 2 assays	NTP, 2007c
	α-Methylstyrene	negative 1 assay; equivocal 1 assay	NTP, 2007b
	Coumarin	negative 2 assays	Api, 2001; Edwards et al., 2000
	Ethylbenzene	negative 2 assays	NTP, 1999; Mohtashamipur *et a*l., 1985
	Naphthalene	negative 1 assay	Harper et al., 1984
	Styrene	positive 2 assays; negative 5 assays	See IARC, 1994
Chromosomal aberrations	Styrene	positive 1 assay; negative 11 assays	See IARC, 1994; IARC, 2002
Sister chromatid exchange	Styrene	positive 6 assays; negative 1 assay	See IARC, 1994; IARC, 2002
UDS	Ethylbenzene	negative 1 assay	Clay, 2001

Table 5. Genotoxicity Studies in Reinforced Plastics Workers

Chromosomal aberrations	IARC	positive 8 studies; negative 12 studies	See IARC, 1994, 2002
	NTP draft	positive 17 studies; negative 13 studies	NTP, 2008
	disagreement	NTP – positive 4 studies; IARC listed as negative	
Micronucleus	IARC	positive 3 studies; negative 10 studies	See IARC, 1994; 2002
	Since IARC	positive 2 studies; negative 2 studies	Vodicka et al., 2004; Godderis et al., 2004; Teixeira et al., 2004; Migliore et al, 2006
	NTP draft	positive 7 studies; negative 10 studies; suggestive 2 studies	NTP, 2008

Sister chromatid exchange	IARC	positive 5 study; equivocal 2 studies; negative 10 studies	See IARC, 1994; 2002
	Since IARC	positive 3 studies	Laffon et al., 2002; Biro et al., 2002; Teixeira et al., 2004
	NTP Draft	positive 9 studies; negative 11 studies	NTP, 2008

Table 4. Summary of *In Vivo* Genotoxicity Studies In Laboratory Animals

Micronucleus	Cumene	negative 2 assays, equivocal 1 assay	Gulf Life Sciences Center, 1985b; NTP, 2007a
	Divinylbenzene	negative 2 assays	NTP, 2007c
	α-Methylstyrene	negative 1 assay; equivocal 1 assay	NTP, 2007b
	Coumarin	negative 2 assays	Api, 2001; Edwards et al., 2000
	Ethylbenzene	negative 2 assays	NTP, 1999; Mohtashamipur *et a*l., 1985
	Naphthalene	negative 1 assay	Harper et al., 1984
	Styrene	positive 2 assays; negative 5 assays	See IARC, 1994
Chromosomal aberrations	Styrene	positive 1 assay; negative 11 assays	See IARC, 1994; IARC, 2002
Sister chromatid exchange	Styrene	positive 6 assays; negative 1 assay	See IARC, 1994; IARC, 2002
UDS	Ethylbenzene	negative 1 assay	Clay, 2001

Table 5

Table 5. Genotoxicity Studies in Reinforced Plastics Workers

Chromosomal aberrations	IARC	positive 8 studies; negative 12 studies	See IARC, 1994, 2002
	NTP draft	positive 17 studies; negative 13 studies	NTP, 2008
	disagreement	NTP – positive 4 studies; IARC listed as negative	
Micronucleus	IARC	positive 3 studies; negative 10 studies	See IARC, 1994; 2002
	Since IARC	positive 2 studies; negative 2 studies	Vodicka et al., 2004; Godderis et al., 2004; Teixeira et al., 2004; Migliore et al, 2006
	NTP draft	positive 7 studies; negative 10 studies; suggestive 2 studies	NTP, 2008
Sister chromatid exchange	IARC	positive 5 study; equivocal 2 studies; negative 10 studies	See IARC, 1994; 2002
	Since IARC	positive 3 studies	Laffon et al., 2002; Biro et al., 2002; Teixeira et al., 2004
	NTP Draft	positive 9 studies; negative 11 studies	NTP, 2008

Table 6. Mouse Lung Tumor MOA Weight of Evidence

Event	Specificity	Biological plausibility	Qual. Human Relevance	Quant. Human Relevance
A. Toxicity results from metabolites generated in lung by CYP2F family				
A.1. Location of cytotoxicity consistent with location of CYP2F	High	CYP2F2 found in Clara cells; cytotoxicity occurs in Clara cells	Limited CYP2F1 in human lung	Cytotoxicity not likely
A.2. Cytotoxicity is consistent across chemicals	High	Cytotoxicity from styrene, ethylbenzene, α-methylstyrene, cumene, divinylbenzene, naphthalene, coumarin, benzofuran	No human lung cytotoxicity reported	Cytotoxicity not likely
A.3. Metabolism is necessary for effect	High	Inhibition of CYP2F2 inhibits toxicity	Limited if any metabolism in human lung	Metabolism not expected
A.4. Species differences in toxicity are consistent with CYP2F. Reactive metabolites are not formed by other CYPs	High	Mice have more 2F2 in lung than rats (2F4); humans have little 2F1; No decrease in lung toxicity in CYP2E1 knockout mice. No effect on lung toxicity by inhibitors of other	Low metabolism indicates low likelihood of tumors	Tumors unlikely

		CYPs.		
B. Cytotoxicity leads to hyperplasia				
B.1. Increased cell replication only in Clara cells	High	Cell death leads to regenerative hyperplasia; cytotoxicity and cell replication both in Clara cells; does not occur in rats.	Hyperplasia not reported in human	Hyperplasia not likely
B.2. Increased cell replication sustained	Medium	Demonstrated through 5 weeks by BrdU; followed by cellular crowding in terminal bronchioles, then hyperplasia; does not occur in rat lung.	Not found	Not likely
B.3 Cytotoxic events (GSH depletion, oxidative stress)	Medium	GSH depletion demonstrated; oxidative stress may be present.	Not found in human tissue	Not likely
C. Sustained cell replication leads to lung tumors				
C1. Late occurring tumors	Medium	Consistent with epigenetic events	Difficult to find among human lung cancer incidence	Not likely
C2. Progression	Medium	Cell replication, leading to hyperplasia	If hyperplasia, then could expect tumors	Not likely

C3. Genotoxic events in lungs	High	Lack of genotoxic responses supports epigenetic MOA	No evidence	Not likely